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The cytoplasmic R391 plasmid interferes with a late repair function in E. coli

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THE CYTOPLASMIC R391 PLASMID INTERFERES WITH A LATE REPAIR FUNCTION IN *E. COLI*

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ABSTRACT

THE CYTOPLASMIC R391 PLASMID INTERFERES WITH A LATE REPAIR FUNCTION IN *E. COLI*

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The R391 drug resistant plasmid increases the UV radiation sensitivity of *Escherichia coli* cells. Results of this study and other efforts indicate that R391 affects a late step in postreplicative repair through the interference of RuvABC function. Results also indicate that R391 does not exist integrated into the bacterial chromosome.

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INTRODUCTION

Drug resistant plasmids (R plasmids) which increase the UV survivability of their bacterial hosts while increasing the level of UV mutagenesis have been studied for over 25 years. Whereas this phenotype confers a selective advantage to its host, plasmids that sensitize its host to UV would appear to confer a disadvantage. Only the plasmid R391 and others in the Incompatibility group J (IncJ) exhibit this unique UV phenotype. In an effort to characterize the UV sensitizing function of R391, many researchers have attempted to isolate R391 plasmid DNA. In all but one, these attempts failed. Thus it has been postulated that R391 exists, for at least part of the time, as an integral part of the bacterial chromosome.

The purpose of this study was to characterize further R391's effects on UV survival in *Escherichia coli* and to determine how it exists in a cell. These studies investigated: 1) the dependence of R391's UV sensitizing function upon genes involved in SOS and recombination DNA repair, 2) the ability of R391 to suppress the DnaA phenotype, 3) the ability of R391 to mobilize chromosomal markers, and 4) the ability of R391 to be transduced into a *recA* host.

LITERATURE REVIEW

Plasmids are autonomous genetic elements found in most groups of bacteria and in some species of yeast. They are most often double-stranded DNA, vary enormously in size, and are capable of existing in three cellular states. They can exist as an extrachromosomal circular molecule, as a cytoplasmic linear molecule (Kinashi et al., 1987), or after integration into the host's genome, as a chromosomal linear molecule. Some bacterial plasmids have the ability to promote their own transfer between cells in a population by a number of different processes including conjugation, transduction, transformation and other less defined modes of gene transfer (Low and Porter, 1978). Bacterial cells which acquire plasmid DNA can adapt to a wide variety of conditions because plasmids often affect cellular metabolism, produce toxins and bacteriocins and provide resistance to heavy metals, antibiotics, and other lethal external agents including ultraviolet (UV) radiation (Howarth, 1965; Strike and Lodwick, 1987).

The major biological effect of UV irradiation is primarily due to its absorption by nucleic acid molecules and the photochemical alteration in structure which results.

This alteration is caused by the formation of pyrimidine photoproducts including cyclobutane-type dimers, pyrimidine-pyrimidone (6-4) photoproducts, spore photoproducts, pyrimidine hydrates, thymine glycols, DNA cross-links and strand breaks (Harm, 1980; Patrick and Rahn, 1976). The most important of these, the cyclobutane-type dimers and the pyrimidine-pyrimidone (6-4) photoproducts, are composed of adjacent pyrimidine residues covalently linked together to form dimers. Cyclobutane-type dimers, or pyrimidine dimers, are the most abundant UV-induced photoproduct (Brash and Haseltine, 1982). They are created when the fifth and sixth atoms of two adjacent residues become joined together resulting in a cyclobutane ring structure (Beukers and Berends, 1960, 1961; Wang, 1960, 1961). Initially, it was reported that the bulky cyclobutane ring introduced significant duplex distortion so that it prevented hydrogen bonding with an incoming base during DNA replication (Chan et al., 1985; Hayes et al., 1971; Hruska et al., 1975). More recent evidence suggests that a single *cis-syn* thymine-thymine dimer, the predominant configuration generated in B-DNA (Patrick and Rahn, 1976), may not produce gross structural changes to B-DNA (Kemmink et al., 1987; Rao et al., 1984; Taylor et al., 1990) and hydrogen bonding to adenine may still be possible, although the bonding strength is reduced (Kemmink et al., 1987). Even though these dimers may be able to participate in weakly bonded Watson-Crick

base pairs, the local alterations in structure they produce are inhibitory to DNA polymerases to the extent that they cause an arrest in DNA replication (Chan et al., 1985). Bending of the local structure surrounding the *cis-syn* dimer has been reported to range from 7 to 30° based on electron microscopy and molecular modeling studies (Husain et al., 1988; Pearlman and Holbrook, 1985; Wang and Taylor, 1991). Such changes to the local DNA environment can have a profound effect on sequence specific DNA-protein interactions, a crucial part of gene regulation and other processes which require cooperative binding to a DNA site. Like pyrimidine dimers, pyrimidine-pyrimidone photoproducts, or (6-4) lesions, occur between adjacent pyrimidine residues; however, the covalent linkage is formed between the C-6 position of one residue and the C-4 position of the adjacent residue (Franklin et al., 1985; Rycyna and Alderfer, 1985). This photoproduct produces severe distortion of the double helix (Taylor et al., 1988) and significantly inhibits DNA polymerases (Chan et al., 1985) without the formation of a cyclobutane ring. To a great extent, unless the original nucleotide structure and sequence is restored by DNA repair systems, functional integrity, and ultimately, cell viability will be jeopardized by UV-induced damage.

Wild type *Escherichia coli* bacteria respond to dimer formation and other UV-induced photoproducts by employing DNA repair systems which either directly reverse or excise the damage. Systems which directly reverse the damage tend to be the simplest systems whereby a single step reaction is catalyzed by a single enzyme. Direct reversal of pyrimidine dimerization occurs by the light-dependent process of photoreactivation. This exquisitely specific system utilizes DNA photolyase (Sancar, 1994; Sancar and Rupp, 1983; Sancar et al., 1984), coded for by the *phr* gene (Harm and Hillebrandt, 1962; Sancar and Rupert, 1978; Youngs and Smith, 1978), and a photon of light to catalyze the monomerization of *cis-syn*-cyclobutyl pyrimidine dimers (Rupert, 1962a, 1962b; Rupert et al., 1958) and to a lesser extent *trans-syn*-cyclobutyl dimers in a single step (Kim et al., 1993). All other *E. coli* DNA repair systems which do not require light are considered to be dark repair systems. It has been empirically determined that for wild type *E. coli* cells, 50-70% of all UV lesions are repaired by photoreactivation, for dark-repair deficient cells, 80-85% of all lesions are repaired by photoreactivation and for the phage T4 which has an excision repair system specific for cyclobutane dimers, only 30-35% of all UV lesions are repaired by photoreactivation (Harm, 1980).

Excision repair is composed of two multi-enzyme systems of repair in which DNA damage is preferentially excised from the transcribed strand (Mellon and Hanawalt, 1989) and the original sequence is restored by DNA synthesis and covalent ligation (Friedberg et al., 1995). These systems are differentiated by the type of damage that is recognized and the excision process. Nucleotide excision repair (NER) is the most general of all excision repair systems and the most effective for UV irradiated photoproducts. This system was once thought only to repair distortive bulky base adducts produced by an array of chemical and physical mutagens including UV irradiation (Van Houten, 1990); however, enzymes crucial to NER substrate recognition do recognize lesions considered not to be distortive (Lin and Sancar, 1989; Pierce et al., 1989; Pu et al., 1989; Tang et al., 1989; Voight et al., 1989).

In *E. coli* it is initiated by the formation of a $(UvrA)_2(UvrB)$ complex, binding of the complex to DNA (Orren and Sancar, 1989) and the unwinding of the DNA duplex (Oh and Grossman, 1986). It has been suggested that the complex "senses" the dynamic state of the duplex as it is unwound and considers it to be damaged if it exceeds the conformational states of a normal duplex (Claassen and Grossman, 1991; Lin and Sancar, 1989; Pu et al., 1989; Van Houten , 1990; Walter et al., 1988). Van Houten and Snowden

(1993) alternatively suggest that the complex may be able to differentiate between normal and abnormal base stacking where a determination of abnormal would initiate the excision process. Once the damaged site is recognized, the dimer of UvrA dissociates (Bertrand-Burggraf et al., 1991; Orren and Sancar, 1989), leaving UvrB stably associated with the DNA at the site of the damage (Orren and Sancar, 1990). UvrC has a high affinity for *uvrB*-DNA associations and once bound, incisions on both sides of the damage (Rupp et al., 1982; Sancar and Rupp, 1983) are produced by the UvrB-UvrC complex (Bertrand-Burggraf et al., 1991; Orren and Sancar, 1989). After the incision, the oligonucleotide fragment and the UvrC protein are displaced by DNA helicase II (Orren et al., 1992), coded for by the *uvrD* gene. *uvrD*, *uvrE*, *recL*, and *mutU* are all alleles of this gene, discovered independently by several investigators who gave it different names (Horii and Clark, 1973; Kushner et al., 1978; Ogawa et al., 1968; Siegel, 1973; Smirnov and Skavronskaya, 1971). UvrB is displaced as the Pol I protein, coded for by the *polA* gene, begins its repair synthesis process utilizing the complementary strand as a template (Orren et al., 1992). The repair is completed as the gap in the phosphodiester backbone is covalently sealed by DNA ligase.

Base excision repair is the second class of excision repair whereby an inappropriate base is recognized by a

group of base specific glycosylases and is then excised. This system is less relevant to the repair of UV-induced photoproducts since the majority of them are bulky adducts. However, thymine glycol-DNA glycosylase (TG-DNA glycosylase) does specifically recognize a minor UV-induced photoproduct, the thymine glycol (Boorstein et al., 1989; Breimer and Lindahl, 1984; Radman, 1976). Repair of thymine glycol lesions begin with their recognition by TG-DNA glycosylase, coded by the *nth* gene in *E. coli* (Asahara et al., 1988; Cunningham and Weiss, 1985). This enzyme sequentially cleaves the N-glycosyl bond between the 5' thymine glycol and the sugar-phosphate backbone followed by the phosphodiester bond 3' to the apyrimidinic site (Kim and Linn, 1988; Kow and Wallace, 1987; Mazumder et al., 1991). Finally, an exonuclease removes the 3' α,β unsaturated aldehyde residue (Doetsch and Cunningham, 1990) so that repair synthesis and ligation can be completed in the same manner as for nucleotide excision repair.

For moderate to large doses of UV irradiation, a bacterial cell may not have the capacity to excise or reverse all of the damage to its genome. One of the most lethal consequences of this unrepaired damage is due to the inhibition of DNA polymerases, and with it, the impairment of chain elongation during DNA replication. In order to deal with the effects of this inhibition, *E. coli* cells

maintain two systems which allow DNA synthesis to continue in the presence of a lesion. In most cases, when DNA polymerase III encounters a lesion, it ceases one nucleotide before the damage (Moore and Strauss, 1979; Chan et al., 1985) and then resumes synthesis approximately 1000 nucleotides downstream (Iyer and Rupp, 1971; Johnson and McNeill, 1978; Smith and Wang, 1989). The gap that results in the newly replicated strand is repaired by daughter-strand gap repair in a manner which does not appear to result in mutations, and hence, daughter-strand gap repair along with excision repair are considered to be the major components of *E. coli's* error-proof repair. With increasing levels of DNA damage, more extreme measures are employed in which fidelity is sacrificed for increased survival. In this case, a modified DNA polymerase is able to insert nucleotides opposite a damaged template with relatively little information as to which nucleotide should be inserted. This mechanism, termed translesion synthesis, appears to be the source of most UV-induced mutations and is the main part of *E. coli's* error-prone repair (Friedberg et al., 1995). These two mechanisms and that which repairs double-stranded breaks do not physically repair the original UV lesion, but instead, deal with secondary consequences of the damage. For this reason, they have been classified by Freidberg et al. (1995) as damage tolerance mechanisms, and

not true DNA repair systems. However, many authors refer to daughter-strand gap repair as postreplicative or recombination repair. These damage tolerance mechanisms, along with nucleotide excision repair, are inducible in *E. coli* by an intricately controlled system known as the SOS response.

The SOS system is initiated in response to the international distress signal of the cell, single-stranded regions of DNA which often result from the arrest of replication (Salles and Defais, 1984; Sassanfar and Roberts, 1990). The RecA protein binds to these single-stranded nucleation sites (Rosenberg and Echols, 1990), forms nucleoprotein filaments (Egelman and Stasiak, 1993) and becomes activated (Salles and Defais, 1984; Sassanfar and Roberts, 1990). Activated RecA initiates the SOS response by facilitating the autodigestion of the LexA repressor (Little, 1984, 1991; Slilaty and Little, 1987) which is bound as a dimer to regulatory regions within SOS inducible genes, or SOS boxes (Kim and Little, 1992; Schnarr et al., 1985; Thliveris et al., 1991). Once cleaved, the repressor can no longer dimerize and repress the SOS genes.

Since the truncated protein has a lower affinity for DNA than does the intact protein (Hurstel et al., 1986; 1988; Kim and Little, 1992), the truncated protein dissociates from the DNA. The inducible genes are then

transcribed at increased levels and turn on the SOS response (Brent and Ptashne, 1981; Little et al., 1981; Sancar et al., 1982). Since RecA is itself repressed by LexA (Little et al., 1981), the autodigestion of LexA will lead to increased levels of RecA and an increased ability to sense damaged DNA. The more tightly the LexA repressor binds to a gene's SOS box the less likely it will become fully induced with a weak signal. As more DNA damage is sensed, more RecA is activated, more LexA is cleaved, and the genes with LexA tightly bound become fully induced. Since both *recA* and *lexA* are repressed by LexA, the system is able to exhibit a spectrum of intermediate states with varied signal induction. Furthermore, since LexA binds to its SOS box more weakly than it does to the operators of other genes, the system returns to its uninduced state more readily. Operators for genes that play roles in nucleotide excision repair bind LexA weakly, as do operators for genes involved in daughter-strand gap repair and double-strand break repair, so that they are induced early in the response (Peterson and Mount, 1987; Schnarr et al., 1991). Operators for genes responsible for translesion synthesis and for filamentation bind LexA more tightly and so are induced later in the response (Peterson and Mount, 1987; Schnarr et al., 1991). In this manner, systems which result in normal fidelity are employed first before the cell commits to the

full mutagenic response. Table 1 provides a partial list of those genes that are repressed by LexA and that characterize the major thrust of the SOS response.

Table 1. Genes repressed by LexA that are part of the SOS response.

Induced response	Induced genes
Nucleotide excision repair	*uvrA, uvrB, uvrD, polB*
Daughter strand gap repair	*recA, ruvAB*
Double strand break repair	*recA, recN*
Translesion synthesis	*umuDC, recA*
Inhibition of cell division	*sulA*

Daughter-strand gap repair restores the continuity of the daughter strand without introducing mutations into the genome. It is initiated by RecA which, with the possible help of RecO, RecR, and RecF, binds to the gaps that result from the arrest of DNA replication. It is believed that these accessory proteins aid RecA in forming a nucleoprotein filament by reducing the inhibition imposed by single-stranded binding proteins (Umezu et al., 1993).

Activated RecA searches the sister chromatid for a homologous match based on sufficient hydrogen bonding between the sister duplex and the strand with the lesion (Hsieh et al., 1990, Kowalczykowski, 1991; Radding, 1991;

Roca and Cox, 1990; Stasiak, 1992; Stasiak and Egelman, 1988). Once the heteroduplex is aligned, RecA facilitates strand exchange through the gap in the 5'⇒3' direction resulting in the formation of a Holliday junction (West et al., 1983). After strand exchange, RuvA binds to the Holliday junction and targets RuvB to the area (Muller et al., 1993; Parsons et al., 1992; Parsons and West, 1993; Shiba et al., 1993; Stasiak et al., 1994). The RuvAB complex facilitates branch migration past the lesion with the aid of RecA, and possibly RecG (Muller et al., 1993, Roca and Cox, 1990; Tsaneva et al., 1992). After branch migration, RuvC cleaves the junction at the point of strand exchange so that the Holliday junction is resolved (Bennett et al., 1993; Connelly et al., 1991; Dunderdale et al., 1991; Iwasaki et al., 1991) and the repair is completed as a DNA polymerase fills in the resulting gap in the parental strand. Daughter-strand gap repair and the SOS system remain active as long as RecA is active. These systems cease to operate when all preferred nucleation sites for RecA are eliminated. This would require that all gaps be repaired by daughter-strand gap repair and all lesions which result in gaps be repaired by nucleotide excision repair.

UV irradiation does not directly induce double-strand breaks; however, they may occur after two relatively close dimers on opposite strands are excised or after a gap is

generated from the arrest of replication opposite a single-stranded break (Bonura and Smith, 1975; Wang and Smith, 1986). The cell repairs these breaks through recombination mechanisms similar to those used for daughter strand gap repair, except that these cells must be grown in a medium that will support multiple replication forks (Krasin and Hutchinson, 1977). It appears that this repair is initiated as the RecBCD enzyme binds the DNA at the double-strand break and searches for a chi site (5' -GCTGGTGG-3') (Dixon and Kowalczykowski, 1991; Smith and Wang, 1987, 1989; Wang and Smith, 1986). Once found, it uses its exonuclease activity to cut the strand 3' to the chi site. In one postulated mechanism, RecA would then utilize the resulting single-stranded tail to mediate strand exchange with a homologous duplex leading to the formation and resolution of a Holliday junction (West and Howard-Flanders, 1984). Evidence suggests that RecN may be specific to this process and that it is regulated by the SOS system (Lloyd et al., 1983; Rostas et al., 1987; Sargentini and Smith, 1986b, 1988). Other proteins that are involved in recombinational repair of double-strand breaks include RecF, RecJ, RadA and UvrD (Sargentini and Smith, 1986a).

Translesion synthesis is also an SOS-inducible DNA damage tolerance mechanism which ensures daughter strand continuity opposite unrepaired DNA. It occurs when an

altered DNA polymerase is able to insert nucleotides into a growing chain across from a lesion which is a misinstructional template. However, the inserted nucleotides are often not correct, and if they are not, mutations may arise. Some evidence suggests that the polymerase responsible for translesion synthesis in *E. coli* is DNA polymerase III with an alternate form of its β subunit (Tadmor et al., 1992), the subunit responsible for a sliding clamp and ultimately its processivity (Kornberg and Baker, 1992). Other evidence suggests that DNA polymerase III lacking its proofreading ε subunit may be responsible (Ciesla et al., 1990; Foster et al., 1989; Jonczyk et al., 1988). Translesion synthesis absolutely requires the functions of the *umuDC* genes (Elledge and Walker, 1983; Kato and Shinoura, 1977; Sedgwick et al., 1991; Shinagawa et al., 1983) and it has been postulated that they encode either the alternate form of the β subunit or act to inhibit the ε subunit (Foster et al., 1989; Tadmore et al., 1992). The *umuDC* operon, as with other members of the SOS regulon, is repressed by the LexA protein (Kitagawa, 1985; Perry et al., 1985; Shinagawa et al., 1983). However, UmuD is unusual among members, in that like LexA, it is induced by activated RecA to autodigest (Burckhardt et al., 1988; Perry et al., 1985; Shinagawa et al., 1988). Once UmuD is cleaved, the truncated proteins dimerize and become active (Woodgate et

al., 1989). Heterodimers of the truncated and untruncated forms of UmuD are inactive and decrease the cell's ability to turn on the error-prone SOS response (Battista et al., 1990). In addition to UmuCD and RecA, SOS mutagenesis is also dependent upon the chaperone proteins GroEL and GroES which may be responsible for the folding of UmuC (Donnelly and Walker, 1989, 1992; Krueger and Walker, 1984).

The error-prone repair of UV-induced lesions produces a characteristic spectrum of mutations which is dependent on DNA sequence, and hence, its local chemical environment. In *E. coli* approximately 75% of all mutations which arise from UV irradiation tend to be base substitutions, with the majority being G·C ⇒ A·T transitions. Frameshifts (15%), tandem double-base substitutions (4%) and deletions (7%) compose the remainder of the UV-induced mutational spectrum (Livneh et al., 1993). The pyrimidine-pyrimidone (6-4) lesion is one of the most mutagenic of all UV photoproducts. After replication of an M13 vector containing this lesion at a specific site in an *uvrA* defective *E. coli* strain, 91% of the phage contained mutations and, unexpectedly, nearly 93% of all mutations induced were 3′ T ⇒ C transitions (LeClerc et al., 1991). Unlike the pyrimidine-pyrimidone (6-4) lesion, the *cis-syn* thymine-thymine and the *trans-syn* thymine-thymine cyclobutane dimers do not appear to be nearly as mutagenic. By using a similar system, only 7% of

the phage contained mutations after replication across a *cis-syn* dimer and 11% of the phage contained mutations after replication across a *trans-syn* dimer (Banerjee et al., 1988, 1990). The spectra of mutations for the *cis-syn* and *trans-syn* thymine-thymine dimers were less specific than for the highly specific (6-4) lesion. Of the mutations induced by a *cis-syn* dimer 57% were A ⇒ T transversions and 31% were T ⇒ C transitions, with most mutations at the 3′ thymine (Banerjee et al., 1988). In contrast, the *trans-syn* thymine-thymine dimer induced mostly T ⇒A transversions and some T ⇒ C transitions, with most mutations at the 5′ thymine (Banerjee et al., 1990). The results of these studies suggest that without SOS induction, thymine-thymine dimers inhibit the progression of DNA polymerase III. However, with error-prone repair, replication is possible with only a mild reduction in fidelity (Friedberg et al., 1995).

When it was discovered that certain plasmids can increase the survival and mutagenesis of their hosts after UV irradiation, it was of interest to determine how plasmid gene products interacted with host functions to produce this phenotype (Marsh and Smith, 1969; Pinney, 1980). Probably the best studied of these plasmids and one that exhibits one of the largest increases in UV mutagenesis and survival is the deletion derivative of R46, pKM101 (Mortelmans and

Stocker, 1976). Due to this trait, pKM101 has been incorporated into the Ames test for mutagens to provide increased sensitivity (Levine et al., 1994; McCann et al., 1975; McCann and Ames, 1976). Perry et al. (1985) identified the locus responsible for the pKM101-mediated effects and determined that the plasmid-borne proteins MucA and MucB were homologues of the UmuC and UmuD proteins. Like *umuDC,* the *mucAB* operon is regulated by LexA (Perry et al., 1985) and the MucA protein is induced by an activated RecA protein to autodigest (Hauser et al., 1992; Shiba et al., 1990). If the production of UmuC and UmuD are rate-limiting factors, then an increased production of these analogous proteins should result in an increase in the level of SOS-dependent error-prone repair. Thus the Muc UV phenotype can be attributed to the production of the plasmid-associated MucAB proteins with a concomitant increase in translesion synthesis.

Pinney (1980) determined that the Muc UV phenotypes exhibited by pKM101 are not always jointly expressed and that UV protection is not universal among plasmids that exert UV effects. Of twelve plasmids from eight incompatibility groups that increase UV mutagenesis as well as change the UV survival of their hosts, only two did not protect the cell against UV irradiation, and of the two, only R391, a member of the incompatibility group J (IncJ),

sensitizes its host to UV (Pinney, 1980). In addition to its UV effects, R391 encodes resistance to kanamycin and mercury, is conjugative and was derived from a South African hospital strain of *Proteus rettgeri* (Coetzee et al., 1972; Hedges et al., 1975). Others in the incompatibility group J, including R997, R705, R706, R748, and R749, also exhibit the UV sensitizing phenotype, Uvs^{-} (Pembroke and Stevens, 1984).

It then became of interest to investigate the mechanism by which R391 and other IncJ plasmids sensitize their hosts to UV while still increasing UV mutability. Pembroke and Stevens (1984) showed that when irradiated cells deficient in excision repair carrying R391 were allowed to incubate in photoreactivating light, UV survivability was restored to the plasmid strain at levels similar to nonplasmid strains. From these data, it was concluded that the plasmid effect was independent of photoreactivation.

Using bacterial mutants deficient in one or more types of DNA repair, it was demonstrated that the sensitizing function of R391 was independent of *uvrA, uvrB, umuC, recF, polA, uvrD, uvrE, and recL* (Pembroke and Stevens, 1984; Upton and Pinney, 1983). Since the plasmid effect did not appear to act through any of the error-proof repair systems tested, it was suggested that it may be due to an alteration in the cell's SOS-inducible error-prone DNA repair system.

If this is valid, the plasmid effect should be absent in *recA* or *lexA* strains harboring R391. In two studies, the sensitizing effect absolutely required a functional *recA* gene; however, the dependence upon *lexA* was less certain (Pembroke and Stevens, 1984; Upton and Pinney, 1983). In the Upton and Pinney (1983) study, there was a clear *lexA* dependence, whereas the Pembroke and Stevens (1984) study demonstrated no dependence on *lexA*. Furthermore, Pembroke and Stevens (1984) reported that the presence of R391 lowered the recombination frequency between an Hfr strain and a *recA*$^{+}$ F^{-} strain. Pembroke and Stevens (1984) interpreted this reduction in frequency to represent a real block in recombination for those recipient cells that contained R391. In light of these facts, it appeared that the sensitizing function of R391 required the ability of RecA to act mechanistically to facilitate recombination as opposed to its ability to induce the SOS-error prone system of repair.

Using complementation analysis, Upton and Pinney (1983) determined that R391's ability to enhance UV-mutagenesis was due to a production of MucAB-like proteins. They demonstrated that R391 restored UV mutagenesis to above that of a wild type *umuC*$^{+}$ cell when transferred to *uvrB umuC* strains (Upton and Pinney, 1983). In order to explain the sensitizing function of R391, Upton and Pinney (1983)

suggested that the MucAB proteins were functional in their ability to enhance translesion synthesis but were altered so that their repair capacity was reduced. However, Ho et al. (1993) reported that R391 in a *recA718 umuCD* background protects cells from the deleterious effects of UV. The *recA718* allele causes constitutive expression of SOS-induced genes with normal recombination function (Friedberg et al., 1995). This strongly suggests that the UV sensitizing and UV mutagenesis functions of the R391 plasmid act independent of one another and that the repair capacity of the plasmid-associated MucAB-like genes is functional.

In an effort to characterize further R391's UV sensitizing function, Nugent (1981) attempted to isolate open and covalently closed circular (CCC) plasmid R391 DNA. Earlier attempts by Hedges et al. (1975) were unsuccessful, as were later attempts, even though numerous methods were employed (Nugent, 1981; Pembroke et al., 1986). To account for this difficulty, Nugent (1981) suggested that R391 exists in an integrated form, similar to several other plasmids, the best-studied being the F factor of *E. coli* (Hayes, 1968; Jacob and Wollman, 1961). The F factor is a 94 kilobase pairs (kbp) plasmid which stably integrates into the bacterial chromosome through a crossing over process. The resulting Hfr (high frequency of recombination) strain is capable of chromosomal transfer, most often incomplete,

between the Hfr strain and a cell without an F factor (F^-). The F sex pilus of the Hfr cell mediates fusion of the two cells and the subsequent conjugative transfer. Other plasmids which are capable of Hfr formation include ColB, ColV, IncIα, FIV, H1, H2, M and N (Datta and Barth, 1976; Kahn, 1968; Sasakawa et al., 1980). Four lines of evidence which support the hypothesis that R391 exists in an integrated form include the ability of R391 to suppress the DnaA phenotype, its inability to transfer kanamycin resistance genes to *recA* strains, the ability to isolate R391 DNA from chromosomal fractions, and its ability to mobilize chromosomal markers (Nugent, 1981).

Nugent (1981) demonstrated that the presence of the R391 plasmid suppresses the DnaA phenotype of a temperature sensitive mutant which carries it. A functional DnaA protein binds to four highly conserved nine base pair long recognition sequences within *oriC* (Kornberg and Baker, 1992). Its binding allows the assembly of DNA polymerase III into its functional holoenzyme form and the consequential initiation of DNA replication. Temperature sensitive mutants deficient in *dnaA* grow to normal titers when incubated at the permissible temperature 30°C; however at the non-permissible temperature of 42°C their titers are significantly reduced. It has been shown that integrated plasmid DNA initiation loci can suppress many temperature

sensitive *dnaA* alleles (Bird et al., 1976; Chandler et al., 1977). Nugent (1981) suggested that the suppression of the phenotype expressed in her study was due to the initiation of replication at the plasmid origin of replication which was stably integrated into the bacterial chromosome.

Nugent (1981) reported no transconjugants between a host strain containing R391 and a *recA* recipient strain. Since the integration of plasmid DNA involves homologous recombination, and the RecA protein is central to this process, it would be expected that if the plasmid exists only in an integrated state, transferred DNA would be degraded in a *recA* strain and no stable recombinants would be formed. Due to the inability to obtain transconjugants with a *recA* recipient strain, it was concluded that the plasmid was integrated into the bacterial chromosome (Nugent, 1981).

Another line of evidence in support of a chromosomal association is the ease with which R391 is able to transfer chromosomal markers. Nugent (1981) measured conjugal mating frequencies between a donor cell that carried R391 and *thr*$^-$ *leu*$^-$ *lac*$^-$ *ara*$^-$ recipient cells. The mating was allowed to continue for 100 minutes and selection for either one of the chromosomal markers or kanamycin resistance was performed. Nugent (1981) determined that the highest transfer frequency resulted when selecting for kanamycin resistance, then *thr*$^+$,

leu$^+$, *lac*$^+$ and *ara*$^+$. The order of the chromosomal markers, with the exception of arabinose, corresponds to the relative genomic locations of these genes on the bacterial chromosome. These data suggested that the chromosomal transfer began from a point near the kanamycin gene which had been stably integrated near the *thr*$^+$ locus. Subsequently 100 colonies able to utilize threonine were tested for the other markers. All threonine positive colonies were kanamycin resistant, where only a fraction of the colonies were positive for the other markers located further from the proposed integration and transfer site (Nugent, 1981).

The last piece of evidence that supports the view that R391 exists integrated into the bacterial chromosome is the ability of Pembroke et al. (1986) to isolate R391 Tn*10* insertion mutants from a chromosomal fraction. The Tn*10* insertion mutants were detected by hybridization of a Tn*10* probe to blots containing chromosomal:R391 DNA fractions (Pembroke et al., 1986). EcoR1 digests of R391 DNA were cloned into the plasmid pUR222 and those clones that represented insertion mutants of the UV-sensitizing gene were identified by their resistance to kanamycin and mercury and their lack of sensitivity to UV irradiation (Pembroke et al., 1986). The fact that R391 DNA was apparently recovered

from chromosomal fractions lends credence to the view that it can exist in association with the bacterial chromosome.

Recently, Ho et al. (1993) successfully isolated cytoplasmic plasmid R391 DNA by using a Qiagen Q-20 column and from an analysis of its EcoRI digestion pattern estimated its size to be 75 kilobase pairs (kbp). They contend that conventional isolation techniques may have failed because of R391's large size and low copy number. The successful isolation of plasmid DNA does suggest that R391 may be able to exist as a covalently closed circular plasmid in some situations.

Kulaeva et al. (1995) have recently characterized the MucAB analogue of R391, $RumAB_{(R391)}$ (R plasmid umu-complementing). This 2481 nucleotide long operon consists of the $rumA_{(R391)}$ and $rumB_{(R391)}$ gene loci separated by a distance of seven nucleotides. It contains a putative LexA binding site beginning 44 nucleotides upstream from the start of $rumA_{(R391)}$. $RumA_{(R391)}$ has been shown to undergo autodigestion mediated by RecA, characteristic of UmuC and of the other plasmid encoded *umuC*-complementing proteins, ImpCAB and SamAB. Phylogenetic analysis of this protein family showed RumAB to form a new subfamily. $RumA_{(R391)}$ clusters in between UmuD and $RumA_{(R446,R471)}$, while $RumB_{(R391)}$ clusters in between UmuC and the subfamily formed by SamB and ImpB (Kulaeva et al., 1995).

The aim of the current study was to investigate further the mechanism by which R391 sensitizes its host to UV and whether it exists as an extrachromosomal circular plasmid or as an integral part of the bacterial chromosome.

MATERIALS AND METHODS

Bacterial strains

Bacterial strain nomenclature is that of Demerec et al. (1966) and Novick et al. (1976). Table 2 lists the relevant bacterial strains used. The R391 plasmid (Coetzee et al., 1972) which contains genes for kanamycin, was originally obtained from Dr. Kristien Mortelmans. Transfer of the plasmid to recipient strains was performed by conjugation as described below.

Media

Cultures were maintained on tryptone medium consisting of 1.0 percent tryptone (Difco) and 0.5 percent NaCl solidified with 1.5 percent agar, and when applicable, supplemented with the appropriate selective antibiotic (Difco). R391 plasmid presence was monitored by resistance to kanamycin at 50 μg/ml included in the tryptone plates. Phage soft agar consisted of 0.8 percent Bacto nutrient broth powder (Difco)and 0.5 percent NaCl solidified with 0.65 percent agar. Mix B was composed of 0.015 M $CaCl_2$ and 0.03 M $MgSO_4$. R plates consisted of 0.8 percent NaCl, 0.1 percent yeast extract (Difco) and 1.0 percent tryptone (Difco) solidified with 1.2 percent agar and supplemented with 0.2 percent 1M $CaCl_2$ and 0.5 percent glucose at 200

Table 2. Bacterial strains used in this study

Bacterial strain	Relevant Genetic Markers	References
E. coli K-12		
KD1088	*thr leu* Δ (*tonB-trpA,B*) *his arg*	Degnen and Cox, 1974 Fowler et al., 1974
KD1088 R391	*thr leu* Δ (*tonB-trpA,B*) *his arg* R391	Magarian, 1983
KD1088 R391	*leu* Δ (*tonB-trpA,B*) *his arg* R391	This study
KD1088 derivatives	*trpA23 thr leu his arg*	Fowler et al., 1974
	trpA23 thr leu his arg R391	Magarian, 1983
	trpA23 thr leu his arg umuC	Dr. N. Sargentini, Kirksville Medical College
	trpA88 thr leu his arg	Fowler et al., 1974
	trpA88 thr leu his arg recA56	This study
	trpA33 thr leu his arg	Fowler et al., 1974
	trpA33 thr leu his arg recA56	This study
	trpA78 thr leu his arg Sm^R	Fowler, unpublished data
	trpA78 thr leu his arg Sm^R *recA56*	This study
	trpA⁻ *thr leu his arg*	
SR596	F^- λ^- *leuB19 thyA deo(C2) lacZ53 malB45 rha-5 rpsL151* Δ(*uvrB-chlA*)	Dr. N. Sargentini, Kirksville Medical College
SR1160	As SR596 but *recB21*	
SR839	As SR596 but *recA56*	
SR305	As SR596 but *recF143*	
SR840	As SR596 but *recF143 recB21*	
S1270	*dnaA46(ts)* Sm^R	Dr. S. Cohen, Stanford University Medical School
JL1478	*lexA3* λ *sulA::lacZ F' lacI*$^{\phi}$ *lacZΔM15::*Tn*9*	Lin and Little, 1988
JL2301	*lexA300* λ *sulA::lacZ*	Hill and Little, 1988

mg/ml. L-broth contained 1.0 percent tryptone (Difco), 0.5 percent yeast extract (Difco), 0.5 percent NaCl and 0.19 percent glucose. For use with SR designated repair deficient strains, L-broth was supplemented with 100 µg/ml thymine (Wang and Smith, 1983). All dilutions and washings were done with sterile 0.85 percent NaCl. Streptomycin was added at 150 µg/ml and kanamycin was added at 50 µg/ml to the appropriate media for selection. Minimal broth consisted of 0.2 percent glucose and 1µg/ml thiamine-hydrochloride supplemented with minimal salts medium (MM) of Vogel and Bonner (1956).

Sterilization

Mix B, tryptophan, thymine hydrochloride, kanamycin, and streptomycin in solution were filter sterilized through a 0.22 µm membrane filter. The remaining compounds were autoclaved in solution at 121°C and 15 p.s.i.

Transductions

P1 transductions were carried out to transfer the *recA56* allele into several recipient strains and to determine if the R391 plasmid could be transduced into a *recA56* recipient strain. L-Broth overnights of the donor and recipient were grown to saturation at 37°C with constant shaking. The P1 phage lysate was diluted to approximately 10^{-7} p.f.u./ml.

Five-tenths ml of the overnight donor culture, 0.1 ml of the P1 lysate, and 3.0 ml of melted phage soft agar were mixed and poured onto fresh R plates. Following overnight incubation at 37°C, the phage overlay was scraped off the plate into a centrifuge tube containing 0.1 ml chloroform. The plate was then washed with 5.0 ml of sterile L-broth and this solution was added to the centrifuge tube. The solution was gently inverted, centrifuged for 15 minutes, and the supernatant which contained the lysate was transferred to another centrifuge tube with 0.1 ml of chloroform. An overnight culture of the recipient strain was centrifuged for 12 minutes, and the pellet was resuspended in 0.5 ml sterile L-broth. Two-tenths of a ml of the recipient culture, 0.5 ml of the P1 lysate and 0.5 ml of Mix B were mixed. Two controls were also performed. The first control contained only recipient culture and Mix B without P1 lysate and the second control consisted of only P1 lysate and Mix B without recipient culture. Both controls were brought to the same final volume as the actual transductants with L-broth. The tubes were incubated for 20 minutes in a 37°C waterbath without shaking, centrifuged for 12 minutes and finally resuspended in 1.0 ml of sterile minimal broth. One-tenth of a ml of this culture was spread on selective plates. After 48 hours incubation at 37°C, the transductant colonies were counted.

In order to select for R391 transductants, selective plates contained tryptone medium supplemented with kanamycin (50µg/ml). For Trp$^+$ transductants, selective plates contained supplemented minimal medium without tryptophan. Selection of *recA56* transductants followed the method of Csonka and Clark (1980). The presence of *recA56* was verified by its extreme UV light sensitivity.

Conjugations and plasmid detection

Conjugations were done following the protocols of Mortelmans and Stocker (1976) and Fowler et al. (1979). L-broth cultures of the donor and recipient were grown overnight to saturation at 37°C with constant shaking. One ml of the donor and 0.1 ml of the recipient were added to 5.0 ml L-broth and again grown to saturation without shaking. Conjugants of plasmid R391 were selected by plating 0.1 ml of the final overnight onto tryptone plates supplemented with kanamycin (50µg/ml) and another antibiotic to which the recipient cell was resistant. The donor and recipient cultures were individually streaked onto the selection media as controls.

DnaA phenotype suppression

The R391 plasmid was introduced into a S1270 *dnaA46* background by conjugation using kanamycin resistance and streptomycin resistance as selective markers. L-broth overnights of the strain S1270 *dnaA46* with and without the plasmid were grown to saturation in a 37°C waterbath. Each strain was diluted to yield approximately 30 to 300 colonies per plate after incubation at 45°C, the non-permissible temperature. Samples to be incubated at 37°C, the permissible temperature, were diluted 10^{-6}. One-tenth of a ml of the appropriate dilution for each strain was plated onto tryptone plates for each incubation temperature. One set of plates was incubated for two days at 37°C and the other set of plates was incubated for two days at 45°C. Following incubation, viable cell number (as evidenced by colony formation) was determined.

Ultraviolet survival

The selected strain both with and without plasmid R391 was grown overnight to saturation in 5.0 ml L-broth. Three ml. of each strain was then added to a 250 ml flask containing 60 ml of L-broth. The cells were incubated in a shaking waterbath until they reached log phase with a concentration of approximately 1 x 10^{8} cells/ml. For the strains used in this study, these conditions were met when

the optical density as determined by a spectrophotometer was approximately 0.75. Five ml of the log phase cells were spun down and the pellet resuspended in 5 ml of sterile 0.85 percent NaCl. Each strain was diluted so that approximately 30 to 300 colonies per plate were viable after irradiation. Unexposed samples were diluted 10^{-7}. One-tenth of a ml of a diluted culture was plated onto tryptone plates. Exposure from a Sylvania germicidal lamp, with and without UV filter cloth dampening, varied from .29 to 78.0 J/m^2. Plates were wrapped in aluminum foil to eliminate photoreactivation and incubated for two days at 37°C. Following incubation, the number of surviving cells (as evidenced by colony formation) were counted.

Interrupted Mating

The R391 donor strain and the recipient strain were each grown overnight separately to saturation in 5.0 ml L-broth. Three ml of each saturated overnight was added to a 250 ml flask containing 60 ml of L-broth. The cells were incubated in a shaking waterbath until they reached log phase with a concentration of approximately 1×10^8 cells/ml. For the strains used in this study, these conditions were met when the optical density as determined by a spectrophotometer was approximately 0.9. Two and one half ml of the donor and 50 ml of the recipient strains were

added to a sterile flask and placed in a 37°C waterbath without shaking. Every 30 minutes, 5.0 ml of the culture was removed from the flask, vortexed to end the mating, and concentrated ten fold in sterile 0.85 percent NaCl. One-tenth of a ml of the concentrated culture was plated onto selective plates and incubated for two days at 37°C after which time colonies were counted. In order to detect R391 conjugants, selective plates contained tryptone medium supplemented with kanamycin (50µg/ml). Selection for Thr^- recombinants was accomplished with plates containing supplemented minimal medium without threonine.

RESULTS

Effect of R391 on UV radiation sensitivities of repair-deficient mutants

The effect of R391 on the UV sensitivities of *uvrB*, *uvrB recB*, *uvrB recF*, *uvrB recA*, *umuC* and *uvrB recF recB* strains was determined. In addition, the UV survival of *lexA3* and *lexA300* cells with and without R391 was investigated. Strains of *uvrB*, *uvrB recB*, and *lexA300* genotypes were clearly sensitized by R391 with at least two orders of magnitude difference in survival noted at the highest exposure (Figures 1, 2, and 3). In contrast, the UV sensitivity of a *uvrB recA* strain did not appear to be altered by the plasmid's presence (Figure 4). Strains of *lexA3*, *umuC* and *uvrB recB recF* genotypes were sensitized to a lesser degree by R391, showing only one order of magnitude difference in survival at the highest exposure (Figures 5, 6, and 7). Strain SR596 (*uvrB*) was first obtained by this laboratory as SR305 (*uvrB recF*); however, it was later determined by its UV survival to be SR596. Similarly, strain SR1160 (*uvrB recB*) was first obtained as SR596 (*uvrB*). Strain SR305 (*uvrB recF*) suffered a mutation that altered its UV sensitivity during the course of the study, and thus, results have not been reported.

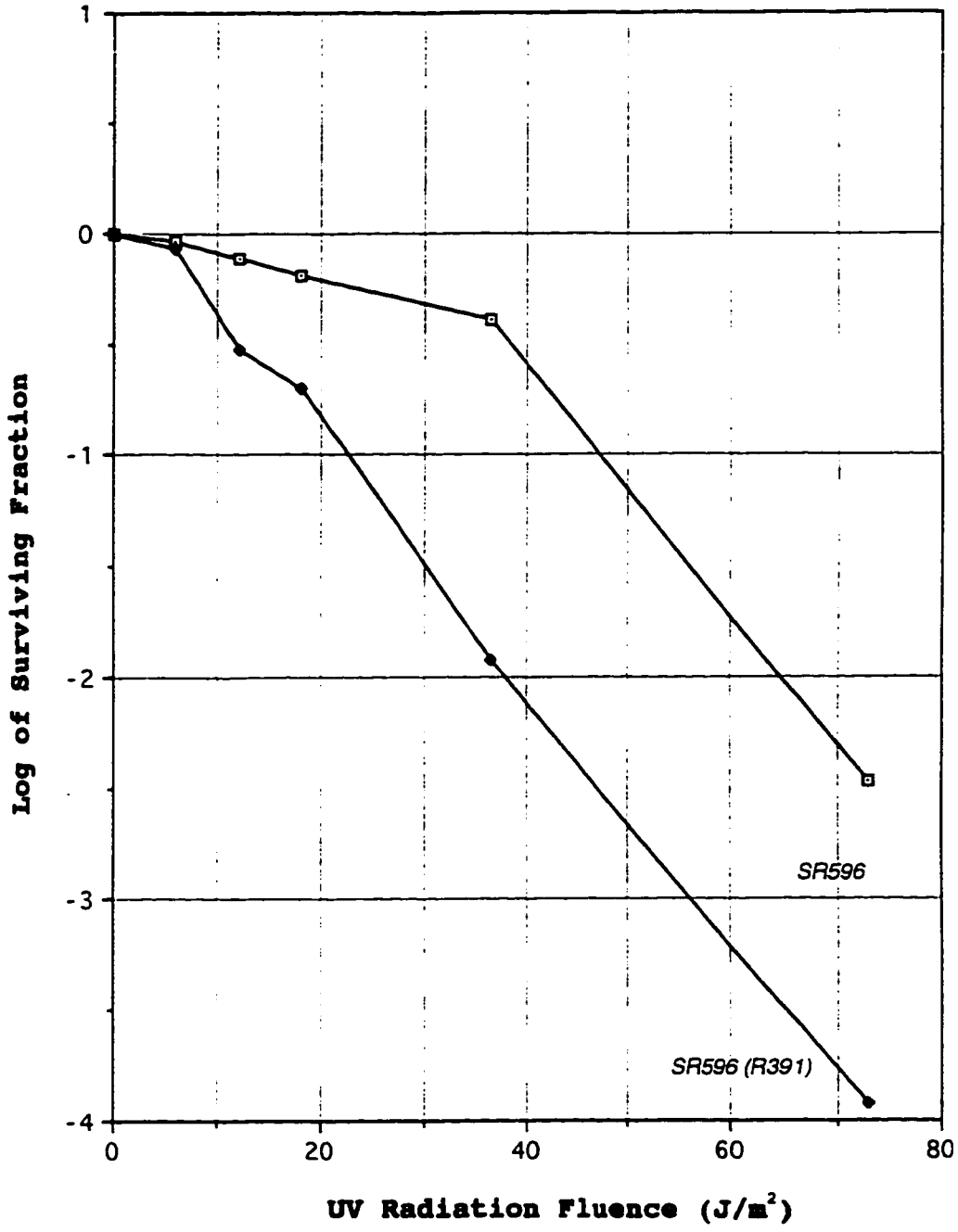

Figure 1. Effect of plasmid R391 on the radiation sensitivity of (SR596) *uvrB*. Data are the average from four experiments.

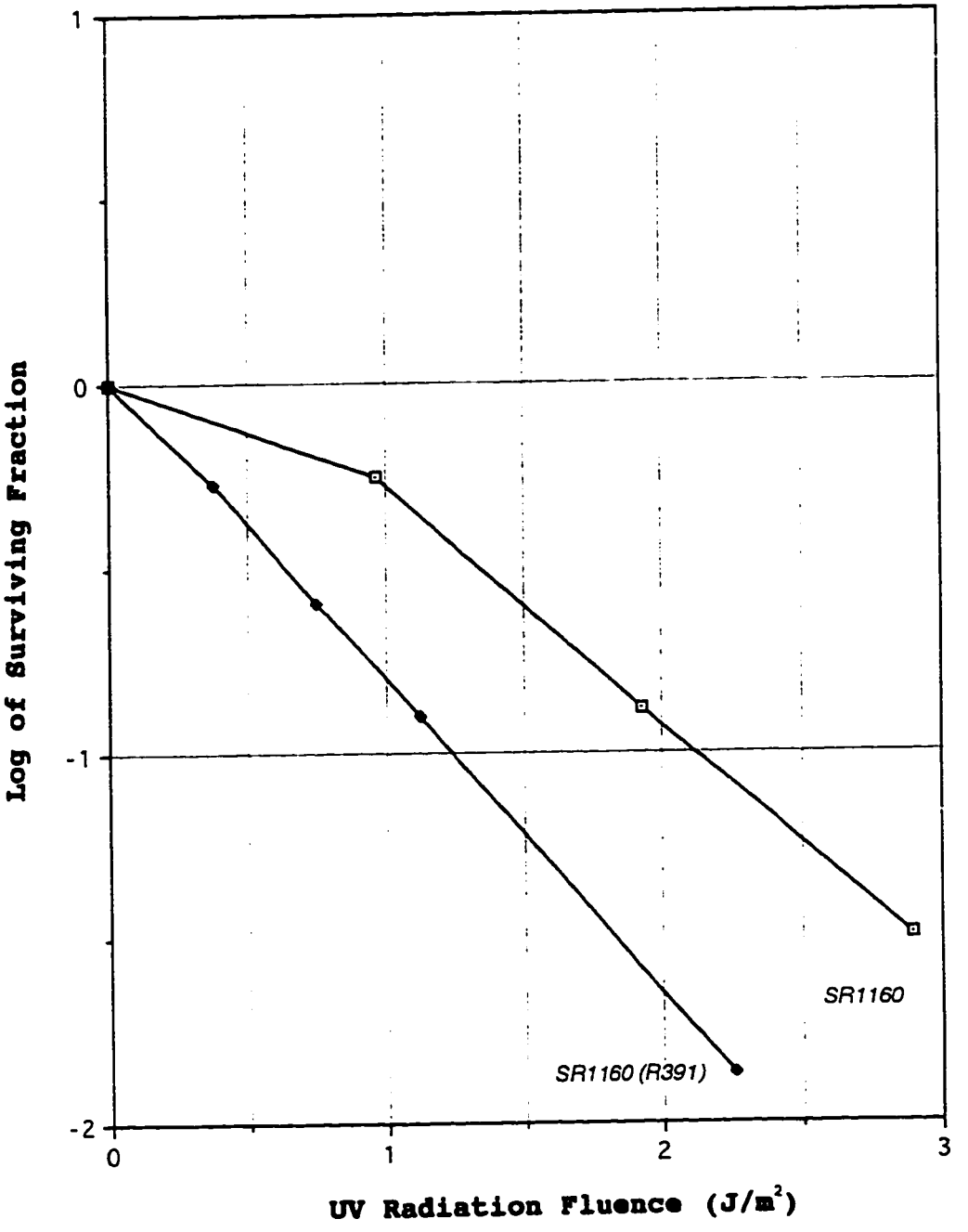

Figure 2. Effect of plasmid R391 on the radiation sensitivity of (SR1160) *uvrB recB*. Data are the average from four experiments.

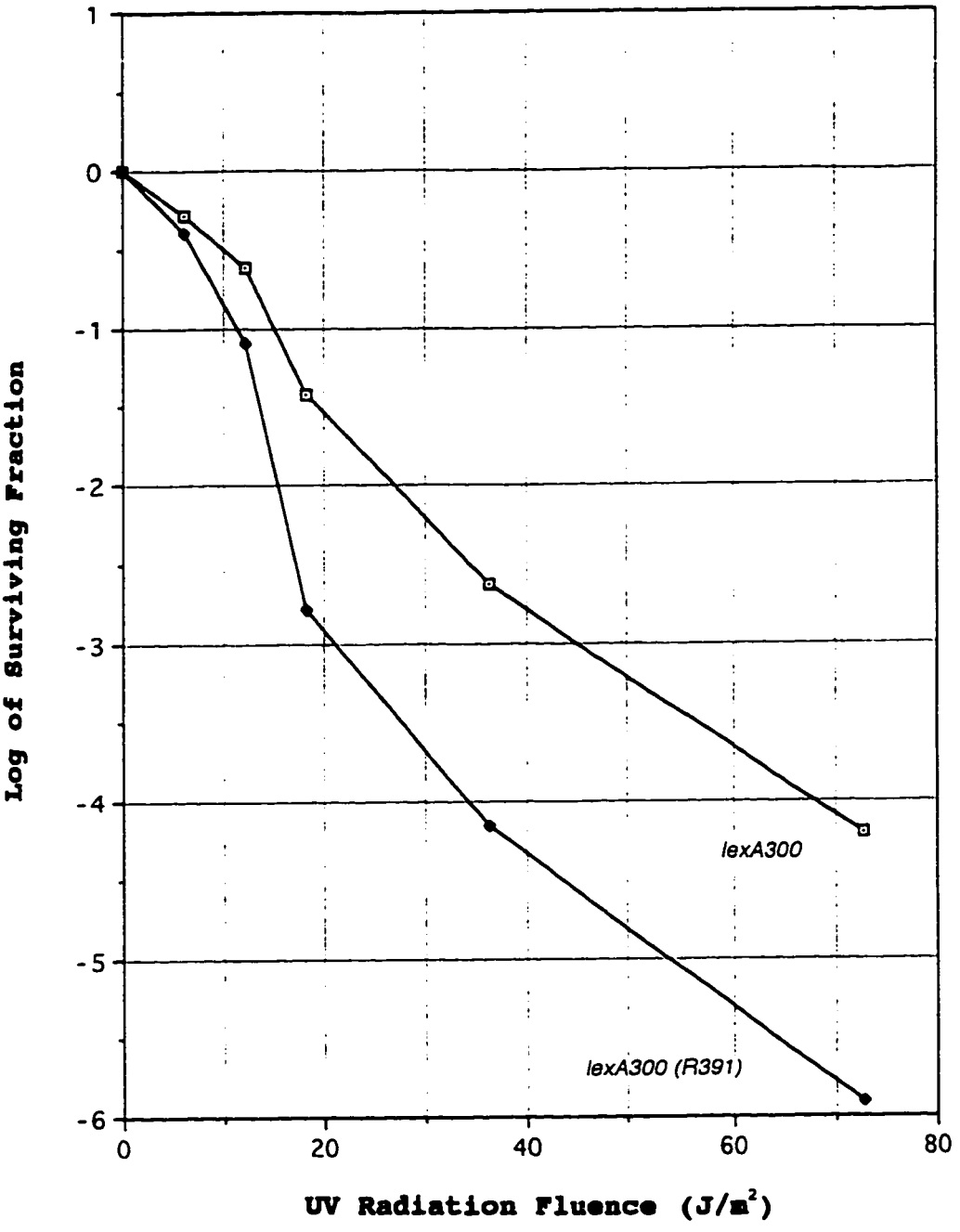

Figure 3. Effect of plasmid R391 on the radiation sensitivity of (JL2301) *lexA300*. Data are the average from two experiments.

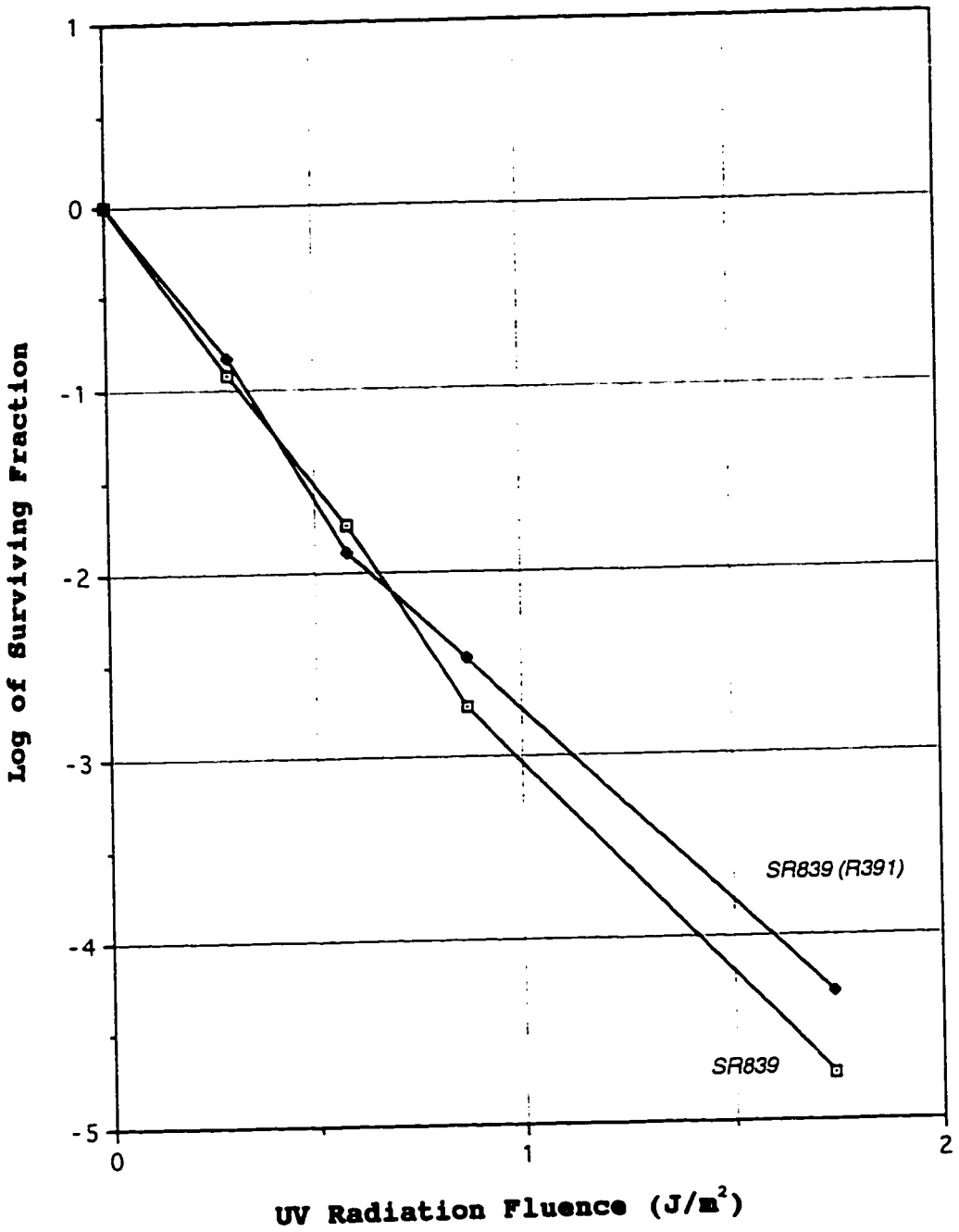

Figure 4. Effect of plasmid R391 on the radiation sensitivity of (SR839) *uvrB recA*. Data are the average from three experiments.

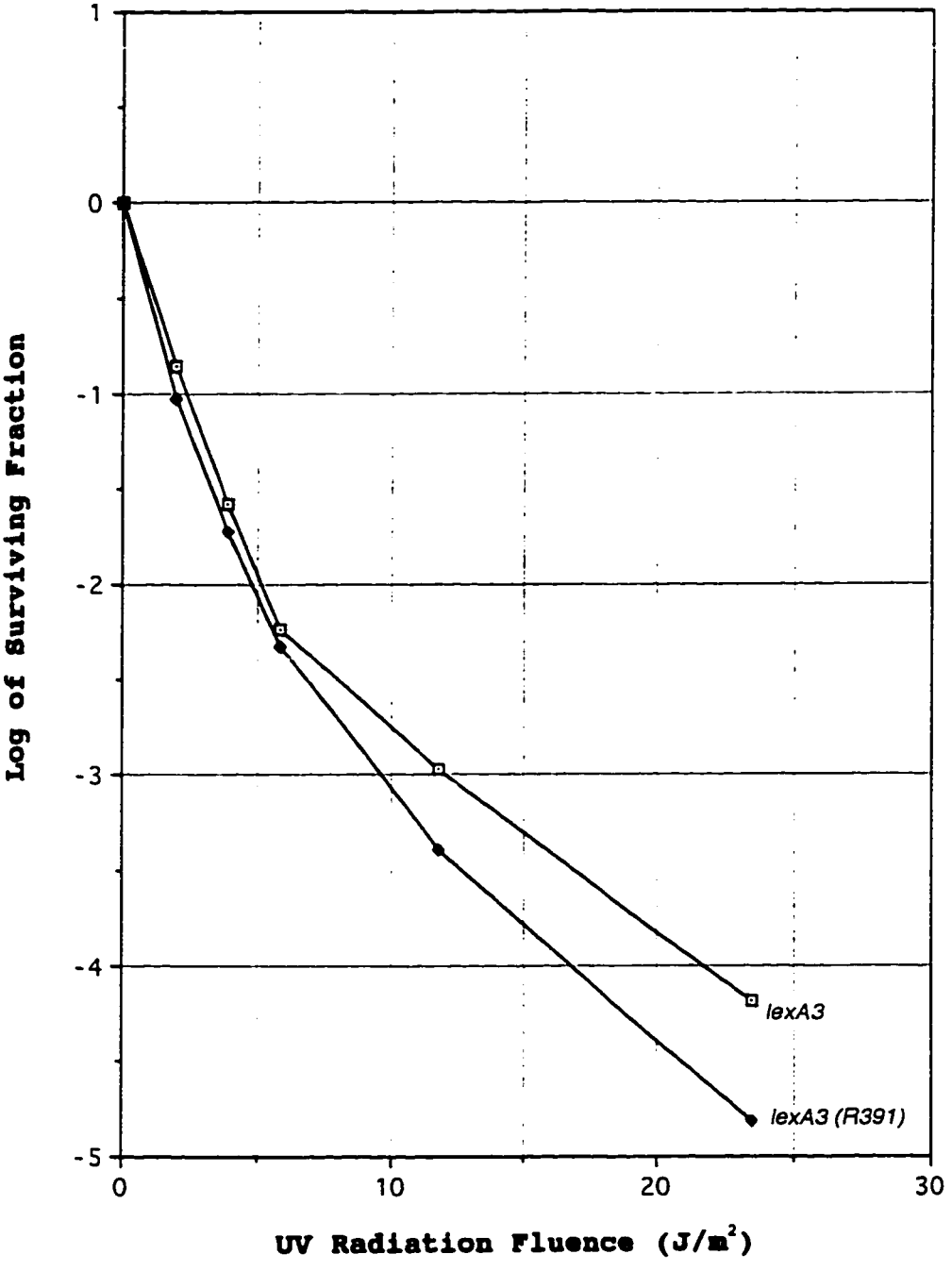

Figure 5. Effect of plasmid R391 on the radiation sensitivity of (JL1478) *lexA3*. Data are the average from three experiments.

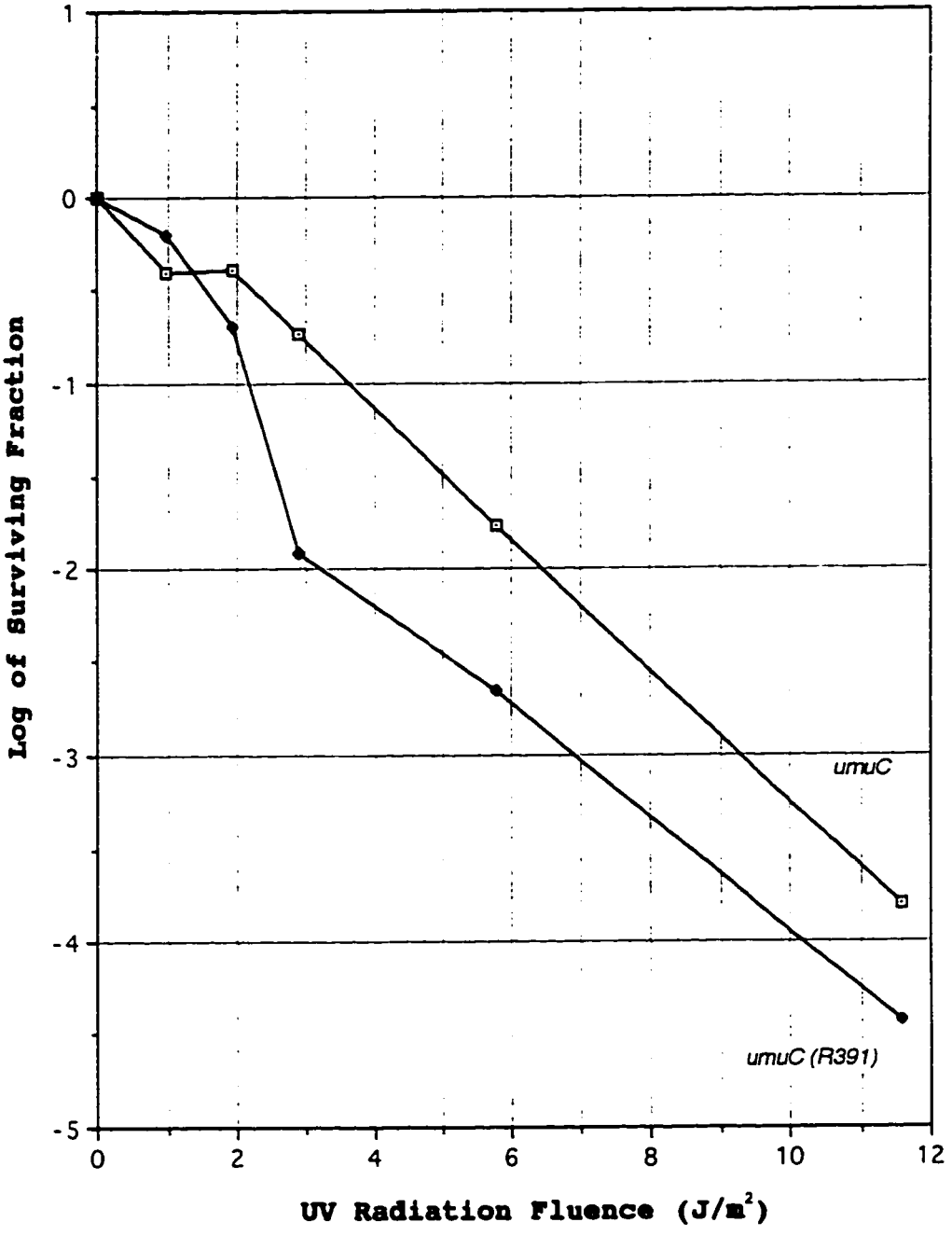

Figure 6. Effect of plasmid R391 on the radiation sensitivity of (KD1088) *trpA23 umuC*. Data are the results of one experiment.

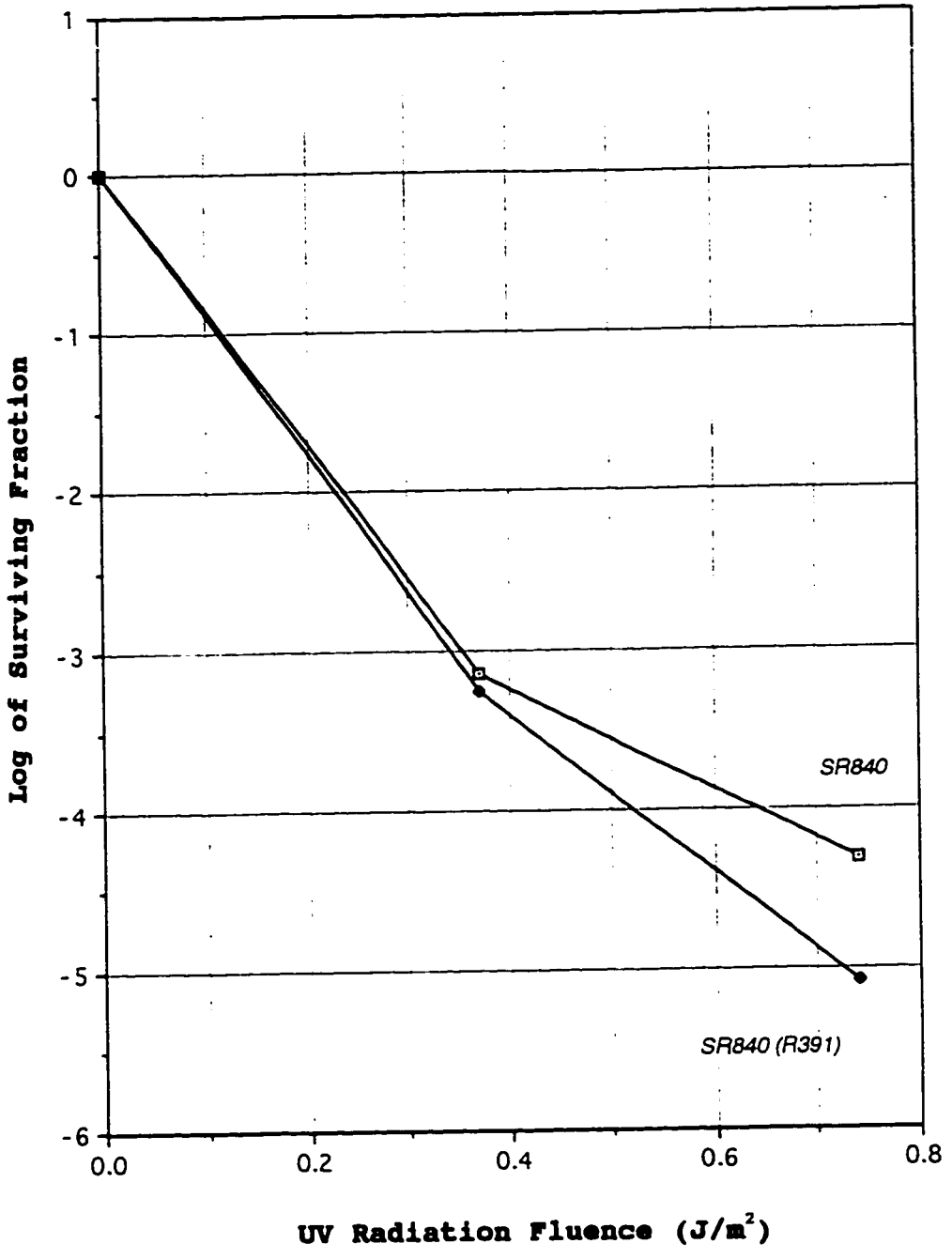

Figure 7. Effect of plasmid R391 on the radiation sensitivity of (SR840) *uvrB recB recF*. Data are the average from two experiments.

Transfer of R391 resistance markers to *recA*$^+$ and *recA*$^-$ strains

The R391 plasmid was transferred to both *recA*$^-$ and *recA56* recipient strains by conjugation and general P1 transduction. The conjugative KD1088 R391 Km^R donor was crossed with *trpA78 recA*$^+$ Sm^R and *trpA78 recA56* Sm^R recipients. Selection was for the kanamycin resistance (Km^R) and for the streptomycin resistance (Sm^R). For this mating, overnight conjugation frequencies were comparable for the *recA*$^+$ and *recA56* strains when Km^R was the selected marker (7×10^{-5} and 1.1×10^{-6}, respectively). There was also little difference between mating times required to first detect a Km^R conjugant for these two strains (one hour and one and one half hours, respectively).

The P1 general transducing phage was used to transfer R391 DNA into *recA*$^+$ and *recA56* recipient strains. The three sets of crosses consisted of KD1088 R391 Km^R x KD1088 *trpA33 recA*$^+$ and KD1088 R391 Km^R x KD1088 *trpA33 recA56*, KD1088 R391 Km^R x KD1088 *trpA78 recA*$^+$ and KD1088 R391 Km^R x KD1088 *trpA78 recA56*, and finally, KD1088 R391 Km^R x KD1088 *trpA88 recA*$^+$ and KD1088 R391 Km^R x KD1088 *trpA88 recA56*. For each set, Km^R transductants were obtained for both the *recA*$^+$ and *recA56* recipients, although more transductants were obtained for the *recA*$^+$ strains than for the *recA56* strains (Table 3). Each of the R391 *recA56* transductants recovered retained

Table 3. Number of transductants obtained for three pairs of recA$^+$ and recA56 recipients with different genetic backgrounds

	Recipient strains					
	KD1088 *trpA33* *recA56*	KD1088 *trpA33* *recA*$^+$	KD1088 *trpA88* *recA56*	KD1088 *trpA88* *recA*$^+$	KD1088 *trpA78* *recA56*	KD1088 *trpA78* *recA*$^+$
KD1088 R391 donor	1[a]	15[a]	2[b]	7[a]	1[c]	11[d]

[a]Number of colonies on ten plates.

[b]Number of colonies on eleven plates.

[c]Number of colonies on twelve plates.

[d]Number of colonies on nine plates.

the ability to act as donors during conjugation with both *recA*$^-$ and *recA56* recipients. In addition, Km^R *recA*$^+$ transductants showed the increase in UV sensitivity conferred by R391 while Km^R *recA56* transductants did not alter the UV sensitivity of their *recA56* hosts. Since these transductants obtained the ability to sensitize *recA*$^+$ hosts, could promote conjugation, and were kanamycin resistant, the genes that encode these functions must total less than 100 kbp, the longest DNA segment that the P1 phage can package (Sternberg, 1990).

In order to evaluate how readily chromosomal markers are transduced into a *recA56* recipient compared to a *recA*$^+$ recipient, the following transductions were performed (KD1088 *trpA*$^+$ x KD1088 *trpA33 recA56*, KD1088 *trpA*$^+$ x KD1088 *trpA88* recA$^+$, and KD1088 *trpA*$^+$ x KD1088 *trpA88 recA56*). Of ten plates examined, there were no Trp$^+$ colonies for the *recA56* recipients. In contrast, over one thousand Trp$^+$ colonies were obtained for the transduction into a *recA*$^+$ strain. Since the recipient *trpA33* and *trpA88* alleles seldom revert (Fowler et al., 1974), it is assumed that the selected Trp$^+$ colonies are true transductants and further that the transduction of the chromosomal *trpA*$^+$ allele into a *recA56* strain is an exceedingly rare event when compared to its transfer into a *recA*$^+$ cell.

Inability to transfer R391 host chromosomal markers to *recA*$^+$ and *recA56* strains

As for kanamycin, the ease with which R391 host chromosomal markers are transferred to both *recA*$^+$ and *recA56* recipients was investigated. For the following crosses, KD1088 R391 *thr*$^+$ x KD1088 *trpA33 thr recA*$^+$ Sm^R and KD1088 R391 *thr*$^+$ x KD1088 *trpA33 thr recA56* Sm^R, Thr$^+$ Sm^R colonies were obtained after a one half hour long mating. For this conjugation, the number of colonies obtained did not seem to increase as the mating time increased and a similar number of colonies were seen for the recipient control after five hours mating. Sixteen Thr$^+$ recipient colonies were purified and all of these were kanamycin sensitive. Thus, there is no evidence that *thr*$^+$, a chromosomal marker, is mobilized by R391 and transferred to a recipient via conjugation. All of the *thr*$^+$ colonies obtained through this mating can be accounted for by the reversion of *thr*$^-$ in the recipient strain.

Inability of R391 to suppress the DnaA46 phenotype

The ability of R391 to suppress the DnaA46 phenotype, a characteristic exhibited by several R factors that integrate into the bacterial chromosome, was investigated (Bird et al., 1976; Chandler et al., 1977). Viable cell number was compared for the S1270 *dnaA46* strain with and without R391

at the permissible (37°C) and the non-permissible (45°C) temperatures (see Table 4). For both the plasmid and non-plasmid containing strain, colony counts at the non-permissible temperature were lower than at the permissible temperature by a factor of 10^5. These data show that the strain of R391 tested did not suppress the DnaA46 phenotype.

Table 4. Viable cell number of strains *dnaA46* and *dnaA46*(R391) at 37°C and 45°C.

Strain	Cell number at 37°C	Cell number at 45°C	Cell number at 45°C/ Cell number at 37°C
dnaA46	2.59×10^{8}	4×10^{3}	1.55×10^{-5}
dnaA46(R391)	2.84×10^{8}	1.2×10^{4}	4.2×10^{-5}

DISCUSSION

R391 is the prototypic Inc J plasmid whose most notable feature is its ability to sensitize wild type *Escherichia coli* to the effects of UV irradiation. Previous studies suggest that this sensitization occurs through inhibition or interference with one or more of the *recA*-dependent postreplicative repair processes (Pinney, 1980; Pembroke and Stevens, 1984; Pembroke et al., 1986). In these studies, this was determined by using cells deficient in a single repair function. Since strains solely deficient in postreplicative repair are likely to perform excision repair to a greater extent than in a wild type cell, these strains might be less likely to show a lack of plasmid sensitization than cells deficient in both excision repair and a postreplicative repair function. For this reason, strains deficient in excision repair with an additional postreplicative repair defect(s) were examined in this study.

Of the two *recA*-dependent postreplicative pathways, daughter-strand gap repair requires functional RecF, RecO and RecR activities, while double-strand break repair involves RecBCD (Friedberg et al., 1995; Tseng et al., 1994). Crucial to both processes, single-stranded binding (SSB) proteins enable RecA to access the damaged strand

while it is maintained in an extended form (Kowalczykowski et al., 1994; Wang and Smith, 1982; West, 1992).

In this study, R391 was clearly shown to increase the radiation sensitivity of *uvrB* and *uvrB recB* strains. Since RecBCD is required to initiate double-strand break repair, it is unlikely that R391 exerts its sensitization function specifically through this postreplicative repair process. In a collaborative effort with Dr. T. Wang of Chang Gung College of Medicine and Technology, Taiwan, R391 was observed to sensitize cells deficient in *uvrB recF* and *uvrA recF* suggesting that R391 does not affect the initiation of daughter-strand gap repair (Wang et al., 1996).

Several plasmids encode their own SSB proteins (Golub and Low, 1986). If R391 were to produce a SSB protein that interfered with the normal function of cellular SSB proteins, recombinational repair may be impaired. However, this was not the case. R391 clearly sensitized *uvrB ssb-113* cells indicating that this ubiquitous process is not affected (Wang et al., 1996). It was also observed that R391 does not affect the synapsis step of recombination as its does not inhibit the repair of daughter-strand gaps in *uvrB* cells (Wang et al., 1996).

Since the plasmid appeared not to affect either the initiation or synapsis step of recombinational repair, it was possible that it exerted its effect through a late step

in recombinational repair. It was observed that R391's sensitization function was clearly dependent upon RuvA and possibly RuvC as R391 did not sensitize *uvrA ruvA60* cells and only slightly sensitized *uvrA ruvC53* cells (Wang et al., 1996). We therefore concluded that R391 affects either branch migration or the resolution of the Holliday junction mediated by RuvABC (Kowalczykowski et al., 1994; West and Connolly, 1992). This model is in agreement with the observed lack of sensitization of the *uvrB recB recF* strain at low UV fluences. When both the *recF* and *recB* recombination repair pathways are blocked, R391 exerts little effect as Holliday junctions are not formed. However at high UV fluences, sensitization by R391 is seen. This is difficult to explain since a *recB recF* strain should have no recombinational repair (Wang and Smith, 1983). The simplest explanation is that an additional recombination repair pathway may exist that has not yet been elucidated. This possibility was previously suggested by Wang and Smith (1983) after they observed that a *uvrB recA* mutant strain is more sensitive than a *uvrB recF recB* strain.

The sensitizing function of R391 seems to be partly independent of SOS regulation. In the present study, we have shown that R391 sensitizes the *lexA300* deletion mutant at all UV fluences and sensitizes the *lexA3* super repressor mutant at high UV fluences. In the *lexA300* cell, the LexA

repressor is not encoded (Hill and Little, 1988) and RuvABC is constitutively expressed at high levels since the *ruvA*˙ *ruvB*˙ operon is part of the SOS response (Benson et al., 1988). R391 sensitization at all UV fluences for a *lexA300* strain is consistent with the model that R391 exerts its sensitization effect through the interference or inhibition of RuvABC function. However, R391 only sensitized the *lexA3* strain at high UV fluences. In this cell, RuvABC is expressed at low levels with the constant repression of the LexA repressor even in the presence of UV-induced DNA damage (Lin and Little, 1988). Since at high UV fluences the plasmid is able to exert some sensitization, the expression of RuvABC must not be entirely dependent on SOS inducement.

Several lines of evidence suggest that the UV sensitizing function of R391 acts independent of its ability to enhance UV mutagenesis. It has been firmly established that the *umuCD* analogues, *rumAB*, are responsible for R391's ability to increase cellular UV mutability (Ho et al., 1993; Kulaeva et al., 1995). In this study, R391 sensitized a *umuC* strain indicating that the sensitizing function is not dependent upon translesion synthesis or error prone repair. Since *rumAB* complement the *umuC* defect, they are thought to affect translesion synthesis. Thus, it is unlikely that the sensitization function of R391 operates through the function of RumAB. This result is consistent with the observation

that cloned *rumAB* genes do not sensitize wild type *E. coli* (Ho et al., 1993). Further, R391 was found actually to protect a *recA718 umuC* strain which is very UV sensitive. Since the *recA718* defect has not been well characterized, it is difficult to interpret this finding. However it suggests that in this background, the sensitizing effect of R391 is abated while the RumAB proteins complement the *umuC* defect. This results in increased DNA repair and increased UV mutagenesis.

In spite of numerous attempts (Hedges et al., 1975; Nugent, 1981), plasmid R391 DNA has been isolated only once (Ho et al., 1993). Due to the inability to isolate plasmid DNA it was suggested that R391 existed as an integral part of the bacterial chromosome (Nugent, 1981). However, in this study I found no evidence for an integrated existence. If R391 required integration, a *recA56* strain which can not carry out homologous recombination, would not function as a recipient for the P1 transduction of R391 because integration of R391 would require recombination (Stanisich, 1988). As a control, a known chromosomal marker (*trpA*$^-$) which absolutely requires integration for stable transduction was transduced into a *recA+* cell at a relatively high frequency. In contrast, no Trp$^+$ transductants were obtained for a *recA56* recipient. This convincingly shows that a segment of DNA which absolutely

requires integration can not be stably transduced into a *recA56* cell where recombination is impaired. Using a R391 strain as a donor, it was possible to obtain Km^R transductants with a *recA56* recipient strain. The frequency of Km^R transductants was lower in a *recA56* recipient compared to a *recA*$^+$ strain but this is not unexpected since *recA56* strains have reduced viability (Csonka and Clark, 1980). Furthermore, all Km^R transductants tested in both *recA*$^+$ and *recA56* backgrounds showed complete R391 function with the ability to promote conjugation and UV sensitivity in *recA*$^+$ cells maintained. This suggests that the entire R391 plasmid was transduced.

It was also possible to readily transfer R391 to *recA56* strains by conjugation. Just as striking, the frequencies of transfer were comparable whether the recipient was a *recA*$^+$ or a *recA56* strain. These results strongly suggest R391 exists in the cytoplasm since recombination would be needed for integration.

Experimental data showed that mating times required to transfer kanamycin resistance to either a *recA*$^+$ or a *recA56* recipient strain were similar, again indicating no evidence of recombination being needed in recipient strains.

There is no evidence that chromosomal markers were mobilized by R391. For the cross between a *thr*$^+$ R391 donor and a *thr*$^-$ *recA*$^+$ recipient strain, only *thr*$^+$ revertants were

obtained with no evidence of *thr*$^{+}$ recombinants. This was also the case for the *thr*$^{-}$ *recA56* recipient. If R391 were integrated, it should be in relatively close proximity to the *thr*$^{+}$ locus on the chromosome (Nugent, 1981) and one would expect to obtain several true *thr*$^{+}$ recombinants for the *recA*$^{+}$ recipient and no recombinants for the *recA56* recipient. The fact that there were only a small number of *thr*$^{+}$ colonies with both the *recA*$^{+}$ and *recA56* recipients indicates these only represent *thr*$^{+}$ revertants and no chromosomal transfer has occurred.

The presence of R391 did not appear to suppress the DnaA46 phenotype. Several R factors that integrate into the bacterial chromosome have been shown to suppress the temperature sensitive DnaA phenotype (Bird et al., 1976; Chandler et al., 1977). At the non-permissive temperature, DNA replication is initiated from the plasmid origin of replication instead of from *oriC* with the residual activity of DnaA in a temperature sensitive mutant sufficient for replicon assembly. If R391 were to be integrated into the chromosome, DNA replication in a temperature sensitive mutant should have preceded from the plasmid origin which would result in comparable numbers of colonies obtained for the permissible and non-permissible temperatures. This was not the case for the R391 *dna46* strain as the number of colonies obtained for the non-permissible temperature were

10^{-5} fold lower than for the permissible temperature. The fact that R391 did not suppress the DnaA46 phenotype in this study further suggests that R391 is not integrated. These data are in contrast with the results of Nugent (1981) who found that R391 did suppress the *dnaA3* allele.

All data from my study support the conclusion that plasmid R391 DNA exists as an extrachromosomal molecule that does not require integration into the bacterial chromosome. The fact that these results are directly opposite to those from earlier work (Nugent, 1981) is surprising and deserves an explanation. It seems most reasonable to assume that R391 can or could exist both as a cytoplasmic plasmid and integrated into the host chromosome. Possibly, isolates of R391 used in this study and another recent study (Ho et al., 1993) have lost the ability to integrate into the host chromosome. Ho et al. (1993) have managed to isolate cytoplasmic R391 DNA although the purification was limited and difficult. They estimated the size of the R391 plasmid genome to be approximately 75 Kbp. This is consistent with the results from this study which showed that the P1 phage may be able to transduce the entire plasmid.

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